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**A new scenario for
the early RNA world**

A. V. Vlassov

Mini-ribozymes and freezing environment: a new scenario for the early RNA world

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Abstract

The RNA World hypothesis states that the present-day life, which is based on DNA genomes and protein enzymes, was preceded by a simpler life form based primarily on RNA. During this era, the genetic information resided in the sequence of RNA molecules and the phenotype derived from the catalytic properties of RNA. Though it is a widely accepted scenario, a number of problems remain unsolved. One of the biggest questions is how complex RNAs could evolve, survive and replicate under typically assumed “warm and wet” conditions, taking into account that the RNA phosphodiester backbone is chemically unstable under these conditions. We suggest that prebiotic conditions associated with freezing could have been of key importance in the early RNA World, and discuss the role of primitive catalytic RNA in the evolution of RNA size and complexity.

1. Introduction

How did life begin on Earth? – Humankind has asked this question continuously for many hundreds of years. Most likely the answer will be never found since the first stages of evolution on Earth took place about four billion years ago, and even rocks from that era are non existent, having been metamorphosed or subducted (not to mention primitive molecules or fossilized organisms). It should be noted that there is a possibility that the simplest forms of life were brought to Earth on meteorites. Even if true, this still does not answer the fundamental question; it simply shifts the location of where life originated to elsewhere in the universe. Thus, it would be interesting to find potential candidates for the first “molecules of life” and to suggest a scenario of evolution.

Present-day organisms, even relatively primitive organisms such as worms, are made up of many millions of cells. Inside each one of these cells, astronomical numbers of different molecules perform sophisticated tasks to keep the master alive and

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happy. There are three major biological polymers: DNA, the nucleic acid responsible for storage of genetic information; RNA, the molecular cousin of DNA, that was long considered just an intermediate molecule involved in translation; and proteins, the final product, synthesized by ribosomes on the RNA template and performing catalytic and structural functions. One of these molecules was the first to appear on the evolutionary stage, and thus has to be capable of performing multiple tasks alone in primitive cells.

Proteins for a long time were considered to be the first “living matter”. Indeed, proteins are ubiquitous biological catalysts today. Peptides are easier to synthesize in prebiotic conditions (Leman et al., 2004), and amino acids were undoubtedly present on the early Earth (Weber and Miller, 1981). In addition, diamino acids are detectable in meteorites (Meierhenrich et al., 2004). However, the vexing difficulty is that molecular mechanisms for self-replication of proteins do not exist, except through either an RNA-mediated mechanism or highly specialized laboratory settings (Lee et al., 1996).

That leaves the two nucleic acids as the more likely candidates for being the original “molecule of life”. RNA was chosen over DNA because of the potentially wider range of reactions it can perform due to the presence of 2'-OH group in ribose. The hypothesis, born in late 60's (Orgel, 1968), stated that in the early days of evolution RNA was the key molecule, performing both the information-storage and catalytic functions. This hypothesis seemed astonishing at first and was not readily accepted. Indeed, RNA was considered to be just an intermediate molecule, carrying information from DNA to ribosomes. It was only many years later that it became crystally clear that what was known before about RNA was just the tip of the iceberg.

In the beginning of 80's a very important discovery was made: it was found that RNA can catalyze chemical reactions (Kruger et al., 1982) that were earlier considered to be solely attributed to protein enzymes. This was the first experimental proof that RNA could have played an essential catalytic role in early biochemistry. By now, RNA-catalyzed RNA synthesis has been experimentally demonstrated (Joyce, 2004; Yarus, 1999). The combined results of many experimental studies of RNA and observations from modern metabolism are consistent with its derivation from a complex RNA-based

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metabolism (Jadhav and Yarus, 2002). Also, the formation of primitive cell membranes has been demonstrated (Deamer, 1997), and the possibility of RNA-controlled membrane permeability was shown (Vlassov, 2001, 2005). All three translational reactions – amino acid activation, aminoacyl-RNA synthesis and peptide bond formation – can be catalyzed by RNA (Yarus, 1999; Joyce, 2004). Thus, the existence of primitive protocells based solely on RNA seems plausible, and the transition to the modern world can be rather well explained. The RNA World hypothesis (Gilbert, 1986), referring to the era prior to coded peptide synthesis where RNA was the major structural, genetic and catalytic agent, has now become a widely accepted scenario.

Though RNA is the best candidate for an immediate predecessor of DNA and encoded proteins, several unanswered questions remain. One of the biggest problems is how it would be possible for long, complex RNA molecules, which were presumably required to perform multiple complex functions, to be formed and survive under the “warm and wet” conditions commonly assumed to have been present early in evolution? Here we review recent findings that support the alternate, cold-origins view, particularly the ability of RNA to catalyze synthetic reactions under freezing conditions that favor RNA stability, and the role that very small, primitive ribozymes may have played in the evolution of RNA complexity.

2. A big problem of the RNA World hypothesis: RNA instability in “warm and wet” conditions

The naturally occurring catalytic RNA (ribozymes) can be considered relics from the hypothetical RNA world, particularly as the phylogenetic distribution of some ribozymes, most notably ribosomal RNA, is wide (Walter and Engelke, 2002). All the naturally occurring nonribosomal ribozymes catalyze cleavage and ligation of the RNA molecules. The hairpin, hammerhead, hepatitis delta virus (HDV), and Neurospora Varkud satellite (VS) ribozymes are found in viral, virusoid, or satellite RNA genomes and have length of 50–150 nucleotides (Harris and Elder, 2000). Group I and group II introns and

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procaryotic ribonuclease P are even larger (several hundred nucleotides) and more structurally complex ribozymes (Kirsebom, 2002). The invention of in vitro selection (SELEX; Tuerk and Gold, 1990) allowed researchers to successfully isolate far more classes of ribozymes than are preserved in nature (Landweber et al., 1999; Joyce, 2004). As these experiments were designed to probe the range of natural catalytic RNA, most selected molecules derive from libraries of 50–100 random nucleotides, and thus the active molecules are the same length, although their functional domains may be smaller. It is a challenge to imagine how such long RNAs could emerge and replicate under typically assumed warm aqueous prebiotic conditions, taking into account that the RNA phosphodiester backbone is chemically unstable in solution, undergoing cleavage through transesterification (Pace, 1991). The cleavage reaction is accelerated by the presence of di- or multivalent metal ions ($M^{\geq 2+}$), the same cofactors that are usually considered vitally important for RNA catalysis (Kazakov, 1996). Unchecked, this cleavage reaction would clearly limit the length and complexity of RNA that could be made via spontaneous polymerization (Pace, 1991).

The problem of RNA instability would be solved if smaller RNAs that could be generated by random polymerization can have synthetic activity, particularly the ability to ligate other short fragments into larger RNAs (Fig. 1). The existence of such ligases provides a much more efficient path to the formation of more complex RNAs than stepwise polymerization (Schmidt, 1999). It is not hard to envisage random polymerization of activated mononucleotides (Monnard et al., 2003) creating small fragments with some catalytic activity, followed by assembly of larger RNAs by ligation. However, small ribozymes may only support simple reactions (like cleavage and ligation) with modest rate enhancements, while more complex reactions (like polymerization and peptide bond formation) requires larger ribozymes. Thus, it would be helpful to provide a unique environment (composition of solvent, surface, temperature) that favors RNA stability over hydrolysis.

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3. Natural and in vitro selected mini-ribozymes

There are numerous versions of ribozymes that occur naturally in the structures of long RNAs. Dange et al. (1990) found that a 31 nucleotide RNA, which is normally excised from the 5' end of the *Tetrahymena* group I rRNA intron during autocyclization, can further undergo self-cleavage at a specific site in the presence of Mn^{2+} cations, generating 5'-hydroxyl and 2', 3'-cyclic phosphate termini. More detailed investigation of the Mn-induced specific cleavage of this RNA explored various mutant versions, as well as fragments and shorter derivatives of the 31-mer (Kazakov and Altman, 1992). This work revealed an active complex consisting of only 7 nucleotides (5'-GAAA/UUU-3') that promote specific cleavage between the G and A at 37°C and pH 7.5. Additional nucleotides flanking the oligonucleotides in the minimal complex were unnecessary for the cleavage reaction to take place, but can affect the rate of the reaction. This GAAA/UUU complex is the smallest catalytic RNA system known to date. The cleavage rate enhancement by this mini-ribozyme is very low – about three orders of magnitude less than for the hammerhead ribozyme, for example, an optimized product of evolution. This suggests clear trade-offs between size and efficiency. However the smallest molecules are likely to arise first, and any rate enhancement would have been beneficial in the early RNA World. Besides, RNA could use various exogenous molecules (cofactors, coenzymes) to enhance its own catalytic activity (Meli et al., 2003; Jadhav and Yarus, 2002).

In vitro selection is the best technique to seek for small RNA catalysts, including ligases that might be, as discussed above, crucial for the early stages of evolution. Landweber and Pokrovskaya (1999) selected a novel class of ligase ribozymes that can append a small RNA substrate molecule to their 5' ends. The surviving molecules in this experiment were minimal 29 nucleotide RNA ligases that require only two selected nucleotides for function, in the context of a series of apparent conventional base pairing interactions. A consensus sequence within the random region provided a template to position the 2'-hydroxyl of the substrate RNA near the 5' triphosphate of the pool RNA.

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This extreme simplicity – requiring only a single bulged nucleotide in a mostly paired RNA duplex – suggests that such a motif would have likely been accessible in a pool of RNA sequences more than 3 billion years ago during prebiotic evolution. Interestingly, it turned out that the catalytic core selected from random sequences contained precisely the seven nucleotide manganese-dependent self-cleavage motif present in the *Tetrahymena* group I intron (Kazakov and Altman, 1992), making this mini-ligase the first dual-catalytic RNA isolated from purely random sequences. The RNA promotes both ligation at the selected site and cleavage at another, with possibly two folded conformations surrounding a divalent metal-ion binding pocket. Because the second activity (cleavage) arose during the experiment as a by-product of selection for ligation, and because manganese was never provided during the original experiment, these results provide an experimental RNA demonstration of Darwinian preadaptation. The final product of artificial evolution in this case was ultimately suited for a second catalytic function in addition to the one for which it was selected, but this second function only revealed itself when presented with an altered chemical environment providing Mn^{2+} .

The occurrence of an unexpected pair of ribozyme reactions in a single RNA molecule suggests that the emergence of new catalytic function can sometimes be a simple response to a changing environment, an event that was presumably common on the early Earth. The emergence of new catalytic function can also be the evolutionary combinatorial product of small existing modules, such as metal ion binding sites. Importantly, the process of breaking and joining RNA sequences that the small RNA catalyzes would also help solve the problem of variation in the RNA world. Cascades of cleavage and ligation, catalyzed by simple molecules, could have led to the production of longer, varied sequences in an RNA world, providing the necessary ingredients for further evolution. This experiment therefore provides a window into some of the most basic steps in early molecular evolution.

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4. Frozen solutions provide a stabilizing environment for RNA

Since the primordial ribozymes are likely to have been inefficient as in the examples described above, the most straightforward way for RNA synthesis to outpace random degradation would be if conditions were available to reduce rates of degradation. Such conditions might include minimal levels of accessible di- or multivalent metal cations ($M^{≥2+}$), reduced temperature, solution conditions that disfavor transesterification, adsorption to surfaces that stabilize phosphodiester bonds relative to cleavage products, or any combination of these (Pace, 1991; Lazcano and Miller, 1996). Although many of these circumstances may have been available at the time of prebiotic evolution, freezing seems to be especially attractive for many reasons. Indeed, a number of investigators have argued that much of the water in the oceans was frozen but underwent periodic melting due to volcanic activity or large meteor impacts (Lazcano and Miller, 1996). Interestingly, Jupiter’s moon Europa and Mars are also thought to contain large amounts of liquid water and ice (Clifford and Parker, 2001) and the possibility of synthetic RNA reactions in freezing aqueous solutions lends some credibility to claims that life could emerge on this planets.

In general, reduced temperatures are known to inhibit most reactions. However, freezing does accelerate some chemical and enzymatic reactions that occur in aqueous solutions, perhaps owing in part to the organization of frozen water as well as concentrating of reactants. Examples include tetramerization of hydrogen cyanide to form diaminomalonodi-nitrile (Sanchez et al., 1966), hydrolysis of penicillin (Grant et al., 1961), dehydration of 5-hydro-6-hydroxydeoxyuridine (Prusoff, 1963), oxidation of ascorbic acid by hydrogen peroxide (Grant and Alburn, 1965), glucose mutarotation (Kiovisky and Pincock, 1966), amino acid modification (Grant and Alburn, 1966), and peptide synthesis (Tōugu et al., 1995). RNA-related examples include chemical ligation of phosphorothioate and bromoacyl oligonucleotide derivatives (Gryaznov and Letsinger, 1993), pyrimidine and purine synthesis from ammonium cyanide (Miyakawa et al., 2002), formation of dinucleotides from adenosine 2', 3'-cyclic phosphate (Renz

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et al., 1971) and synthesis of polynucleotides from phosphor-imidazole-activated mononucleotides (Monnard et al., 2003). Altogether, freezing provides a unique environment, greatly reducing RNA degradation while also concentrating solvents and reducing water activity, that can promote a number of diverse reactions.

5. The hairpin ribozyme: exclusive ligase active in frozen aqueous solution

Since ligation was likely a key primordial RNA reaction, the hairpin ribozyme (HPR) has been chosen as the subject of studies of RNA catalysis under freezing conditions (Kazakov et al., 1998; Vlassov et al., 2004). Under standard solution conditions, the HPR can either cleave substrates to generate RNA fragments with 5'-hydroxyl and 2', 3'-cyclic phosphate termini, or ligate them in the reverse reaction; both reactions require either Mg^{2+} or high concentrations of monovalent ions to proceed (Walter and Burke, 1998). Interestingly, the termini that are ligated by HPR are chemically the same as those formed through transesterification during ordinary (i.e., metal-, base-, or acid-catalyzed) "random" cleavage (Kazakov, 1996) – and thus could have been available on the prebiotic Earth. It was first discovered that in frozen solutions, the HPR promotes efficient Mg^{2+} -independent ligation in *cis* (intramolecular ligation), while the cleavage reaction is very slow (Kazakov et al., 1998). Because intermolecular reactions are more useful from an evolutionary point of view, further studies were focused on freezing-induced ligation catalyzed by the HPR in *trans* (Vlassov et al., 2004) (Fig. 2). The key factors promoting the reaction might include RNA concentration, dehydration, and immobilization on the ice surface; it is hard to draw solid conclusions at the moment. The reaction is relatively insensitive to environmental conditions as long as freezing occurs, underscoring the robustness of the system and increasing the likelihood that conditions on the prebiotic Earth could have supported freezing-induced ligation. Importantly, the natural 3'-5' linkage is the major or only product formed under freezing conditions. The maximum initial rate observed at $-8-10^{\circ}C$ was 0.006 min^{-1} , which is several orders of magnitude lower than rates for the conventional ribozymes acting under standard

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solution conditions, at +37°C. This rather slow rate is the non-avoidable trade-off for the dramatically increased stabilization that freezing conditions provide.

5 No ligation occurred if the solution was supercooled to −10°C without freezing, and ligation was very inefficient in solutions containing 3M NaCl that remain unfrozen at −10°C (Vlassov et al., 2004). This indicates that freezing is absolutely required for the reaction, and high concentrations of salts or low temperatures by themselves are not essential. On the other hand, the reaction is most efficient in the range −4° to −12°C, with rates dropping to near zero for temperatures above −1° or below −20°C. Thus, although the reaction *requires* freezing, the temperature cannot be too cold, presumably due to the need for liquid microinclusions in the ice and sufficient thermal energy to surmount the activation barrier for the reaction. In a prebiotic environment, optimal conditions for the reaction would probably include temperatures slightly below freezing, with fluctuations creating frequent cycles of freezing and melting, as occurs for example with day/night cycles in partially frozen pools of water. Melting and warming of frozen solutions would result in dissociation and redistribution of primordial ribozyme and ligation substrates as well as in refolding of the ribozymes. These cycling conditions were mimicked in the laboratory to see if they could affect ligation yields. Samples were frozen at −10°C, melted and incubated at 37°C, then frozen again, with the procedure repeated two to six times. Freeze-thaw cycles indeed increased the ligation yields up to 3-fold (Vlassov et al., 2004).

At decreased temperature, fewer base pairs should be required for stable pairing between the ribozyme and the substrate sequences, suggesting that the sequence specificity of the ligation reaction may be reduced for freezing-induced catalysis compared to the normal solution reaction. To test whether this is indeed the case, the 5′ and 3′ ligation substrates, 3′-LS and 5′-LS (Fig. 2) were partially randomized at residues that should normally form base pairs with the substrate binding sequence of the HPR (helices 1 and 2) and used in the freezing ligation reaction at −10°C. Products of the reaction were reverse transcribed and PCR-amplified using the flanking fixed sequences as primer-binding sites. Cloning and sequencing revealed that 3′-substrate

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sequences ligated by the HPR all contained a G at the 5'-end where ligation occurred, as is known to be absolutely required for catalysis by the wild-type HPR (Chowrira et al., 1991). However, all other selected positions were variable, with no sequence in common among the clones sequenced. In contrast, for the 5'-substrate most of the successfully ligated sequences matched the original sequence. Thus, under the freezing conditions four base pairs are required to bind the 5'-LS but virtually any sequence can be ligated by the HPR-5'-LS complex. These results with HPR as a model ribozyme provide a clear illustration of the feasibility of RNA catalysis upon freezing.

6. In search of a modern ribozyme's ancestors

It can be expected that at lower temperatures, fewer intermolecular interactions (such as hydrogen bonds) would be required to stabilize complexes from small RNA fragments. Some of these complexes would have catalytic activity and could be selected. To test the hypothesis we used a "reverse-evolution" approach (Vlassov et al., 2004) that involve progressive sequence deletion, fragmentation and mutagenesis of the original, naturally existing ribozyme in order to access related sequences, some of which may resemble its "ancestors". Following this strategy and beginning with the HPR consensus structure (Fig. 2), Vlassov et al. progressively eliminated loops, shortened helical segments, eliminated connecting sequences between the catalytic core and the substrate binding domain, and finally deleted the substrate binding domain. Each derivative was assayed for the ability to ligate pairs of RNA fragments with defined 5'-LS and 3'-LS sequences. The results are shown in Fig. 3. Bisecting the RNA in the catalytic core by cutting the loop at the bottom of helix 4 (structure II) reduced the yield of ligation at -10°C by about half (from 50% to 23%), and reduced the ligation yield at 37°C in the presence of Mg^{2+} from 4% to 1% in standard 14 h reactions. Eliminating helix 4 and connecting the C and G that would otherwise form its first base pair (structure III) had a severe effect, reducing the yield of the freezing reaction to 0.7%. Separating the catalytic core from the substrate binding domain of I (structure IV) re-

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duced the ligation yield from 50% to 10%, and decreasing the length of the sequence that participates in helix 1 from 6 to 2 nt (structure V) further reduced the yield to 1%. Curiously, the complete elimination of the substrate binding sequence (structure VI) still left detectable ligation activity under freezing conditions (0.3%, 6-fold enhanced over a background of 0.05%); however, in Mg^{2+} -containing solution at 37°C, structure VI (as well as III-V) had no detectable activity ($\leq 0.05\%$). It is unclear whether this untemplated ligation results from nonspecific binding of the substrates to the catalytic core or from substrate sequences providing the role of the substrate-binding domain. In the latter case, with low temperature stabilizing weak interactions, one of the substrates might act as a highly mismatched splint to position a separate pair of 3'-LS and 5'-LS substrates for ligation by the catalytic core. Finally, we assayed an HPR variant in which G8 is substituted by U for ligase activity under freezing conditions. Substitution of G8 by any other nucleotide is known to completely abolish ribozyme activity under standard solution conditions (37°C/Mg) (Berzal-Herranz et al., 1993). Indeed, in control experiments at 37°C, neither cleavage nor ligation activity were detectable. However, under freezing conditions this mutant ribozyme was active, displaying ligation yields of 5.6%.

Summarizing, the results show that freezing is uniquely able to relax the sequence and structural requirements for ligation in the case of the hairpin ribozyme. Freezing allows mutated, partially deleted, and fragmented HPR derivatives of minimal size to be efficient catalysts at sub-zero temperatures, despite having greatly reduced or zero activity in solution. In the prebiotic context, freezing-induced concentration could contribute to ligation efficiency by bringing together different RNA fragments to form catalytically-active complexes. Also, freezing provides compartmentalization in the liquid microinclusions trapped in the ice crystal to prevent their dispersal – a function later provided by cells. If lipid-like molecules are present, another method of compartmentalization could come into play: the encapsulation of RNA within lipid vesicles induced by freeze-thaw cycles (Pick, 1981). And most importantly, low temperatures greatly limit degradation of newly formed RNA molecules. In later stages of molecular evo-

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lution, the evolved superior catalysts may have survived transfer into “warm and wet” environments since synthetic reactions will be efficient enough to overcome random degradation.

7. Conclusions

5 One of the opened questions of the RNA World theory is how complex RNAs could evolve and survive on the early Earth, given that the RNA is rapidly degraded under typically assumed “warm and wet” conditions. The recent discoveries of several very small ribozymes and the finding that short RNAs can catalyze ligation of RNA fragments under freezing conditions that greatly inhibit random degradation present an
10 elegant solution to this problem. Random polymerization of activated mononucleotides could create small fragments with some catalytic activity, followed by assembly of larger RNAs by ligation, and then eventually large proficient catalysts emerged, capable of surviving in less protected “warm and wet” environments.

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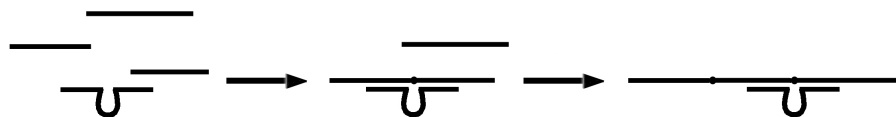


Fig. 1. Step-wise assembly of long RNA polymer from randomly synthesized short fragments by primitive mini-ligase shown schematically.

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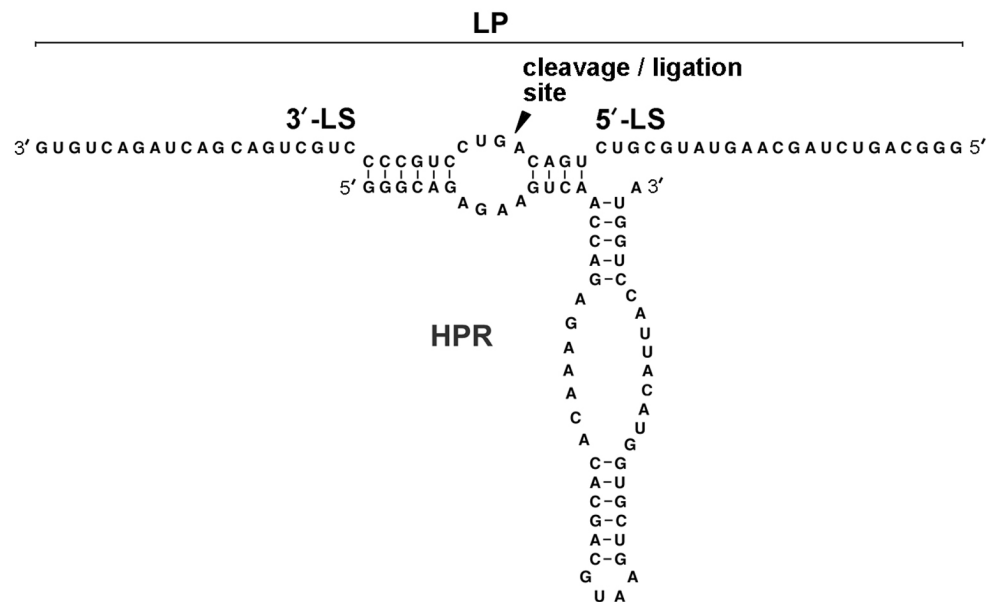


Fig. 2. Structure of the hairpin ribozyme with separated enzyme and ligation substrates. HPR, hairpin ribozyme. 3'-LS and 5'-LS, ligation substrates. LP, ligation product.

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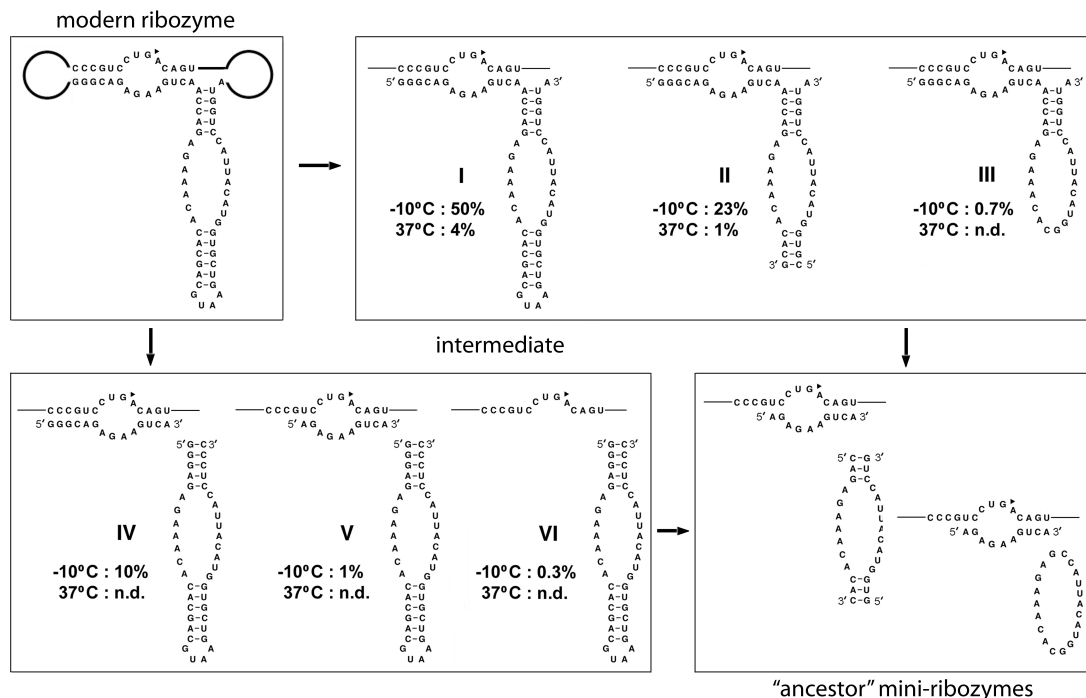


Fig. 3. Scheme of “reverse evolution” of the hairpin ribozyme ligase. The approach involves progressive minimization and fragmentation of the original, naturally existing ribozyme in order to access related sequences that might have included its “ancestors”. Following this strategy, the HPR was simplified (along the directions of the arrows) and several constructs were assayed for the ability to ligate pairs of RNA fragments at -10°C and 37°C : I, HPR; II, bisected HPR; III, truncated HPR; IV and V, HPRs with separated domains; VI, template-deleted HPR. Ligation yields observed after 14 h incubation are shown next to the structures. Ligation substrates 3'-LS and 5'-LS were used in equimolar amounts, and ribozyme-to-substrates ratio was 20:1.

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